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SEARCH FOR THE LESION(S) OF PLATELET STORAGE

Final Report

1970-1976

March 1977

by

Phin Cohen, M. D.

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Harvard University School of Public Health
Boston, Massachusetts 02115

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) We developed techniques for: (1) measuring the lipase activities of human platelets by patterns of release of endogenous fatty acids, as opposed to the release of fatty acids from model substrates; (2) measuring the phospholipase A ₂ activity of human platelets by the release of arachidonic acid as well as by O ₂ consumption; (3) assessing the metabolism of platelets and arteries in vivo. We showed that primate platelets and arteries are incapable of converting lanosterol to cholesterol in vivo; (4) isolating alpha granules and mitochondria from platelet homo-			

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 genates. We ~~attributed our~~ success to two departures: homogenization by cavitation forces developed by explosive decompression, and the elimination of a preliminary centrifugation to remove debris prior to ultracentrifugation. Using this technique, we ~~showed that~~ platelet factor XIII ~~is~~ a cytoplasmic component whereas fibrinogen and platelet factor 4 are concentrated in alpha granules, and platelet factor 3, although present in all fractions was on the basis of phospholipid content, highest in the alpha granule and soluble fractions; (5) measuring the function of mitochondria in the intact human platelet by exposing the platelets to graded decrements of osmolarity. ~~We showed that~~ human platelets are capable of tight respiratory coupling, and are otherwise similar to other mammalian platelets in their metabolic responses in vitro.

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FINAL PROGRESS REPORT: DADA-17-70-C-0083

The work on DADA-17-70-C-0083 from 1 February 1970 - 30 June 1976 developed novel techniques for 1) measuring the lipase activities of human platelets 2) measuring the release of arachidonic acid by human platelets 3) assessing the metabolic capacities of human platelets and primate arteries in vivo 4) homogenizing and subfractionating human platelets 5) examining the function of human platelet mitochondria.

Lipase activities of human platelets measured by patterns of release of endogenous fatty acids Most laboratories use phospholipids labeled with one or two radioactive fatty acids as substrates for the study of phospholipase activity in vitro. This technique has yielded much useful information but has serious shortcomings which center on the artificial presentation of substrate. In the method pioneered by the van Deenen laboratory, the substrate, usually synthesized by liver microsomes and then separated by thin-layer chromatography, must be dispersed by mechanical or chemical means to facilitate its access to catabolic sites. The dispersal, no matter how complete, is not likely to allow the synthesized compound to find a way, in a cellular or particulate system, to precisely mimic the relationship between substrates and enzyme that obtains in vivo.

In the method developed by Bills et al (1976) the substrate is labeled in situ by exposing whole platelets to a labeled fatty acid. This keeps the labeled substrate in its natural position. However, as pointed out by us (Jesse and Cohen, 1976) the distribution of the label among phospholipids may depend more on their anatomical location than their normal propensity to esterify the fatty acid.

In December 1975 (Derksen and Cohen) we described a method for simultaneously measuring the release of all fatty acids from various endogenous substrates. Using this technique, we found four types of lipases in human platelets: two acid (directed towards neutral lipids and the one-position of phospholipids) and two alkaline (directed towards the one-position or the two-position of phospholipids).

Phospholipase A₂ activity of human platelets measured by release of endogenous arachidonic acid and by O₂ consumption We combined the technique of Derksen and Cohen (1975) with polarographic measurement of O₂ consumption and showed that the latter could be used to quantify the release of arachidonic acid by human platelets. We then used the two novel techniques to show the mechanism of the thrombin-mediated burst in oxygen consumption by human platelets (Pickett and Cohen, 1976a), the predilection of phospholipase A₂ in human platelets for diacyl phosphatidyl ethanolamine (Jesse and Cohen, 1976), and the induction of phospholipase A₂ activity in human platelets by trypsin (Pickett et al, 1976), and the calcium ionophore A23187 (Pickett et al, 1977).

We plan to submit sometime in the Spring a final manuscript on work that was completed last year on the effect of thrombin on phospholipase activity by platelets (Pickett et al).

We believe that the new methods for assessing lipase activities will challenge if not displace the techniques that employ radioactivity. Among the several possible lines of investigation that our method is suited for is the assessment of activities of the various lipases during platelet storage. On the one hand, breakdown of triglycerides could provide an energy source; on the other, breakdown of phospholipids could provide free arachidonic acid for synthesis of thromboxanes, endoperoxides and prostaglandins, or could un-stitch the fabric that holds membranes together, thereby causing cell death.

Metabolism of platelets and arteries in vivo In 1973 (Derksen and Cohen) we found that human platelets can synthesize lanosterol and dihydrolanosterol from mevalonate, and cholesterol from desmosterol, in vitro, but are unable to convert lanosterol into cholesterol. We also showed that human coronary artery and aorta are apparently incapable of carrying the label from mevalonate beyond lanosterol. The latter observation conflicted with previous reports describing cholesterol biosynthesis by arterial tissue, possibly owing to differences in technique. To resolve this important issue, we devised novel methods for comparing in vitro and in vivo metabolic capacities of platelets and arteries. In what we described as an in vitro/in vivo method we incubated ^{14}C mevalonic acid with intact human platelets or primate arterial segments in vitro and then returned each to in vivo conditions, the former by transfusion, the latter by reimplantation at the site of removal via end-to-end anastomosis (Derksen et al, 1976). This showed that nonhuman primate platelets and arteries are unable to consummate in vivo the synthesis of ^{14}C cholesterol from ^{14}C lanosterol (the stopping point of the ^{14}C mevalonic acid label in vitro). We then used direct injection of mevalonic acid in vivo to show that the blockade in demethylation of lanosterol did not derive from a biochemical lesion secondary to in vitro manipulation of the tissue. There are two important aspects to these studies. First, they establish that nonhuman platelets and arteries are incapable of in vivo biosynthesis of cholesterol. Second, they describe a technique for assessing the metabolic capacities of blood vessels in vivo, a technique that deserves wider application.

Subcellular fractionation We developed an improved method for isolating alpha granules and mitochondria from human platelets by a swinging bucket density gradient method (Broekman et al, 1974). We attributed our success to two departures: homogenization by cavitation forces developed by explosive decompression, and the elimination of a preliminary centrifugation to remove debris before ultracentrifugation. We then used this new technique to show that platelet factor XIII is a cytoplasmic component whereas fibrinogen and platelet factor 4 concentrate in alpha granules (Broekman et al, 1975) and that platelet factor 3, although present in all fractions, was, on the basis of phospholipid content, highest in the alpha granule and soluble fractions (Broekman et al, 1976a). We then adapted our swinging bucket density gradient method (Broekman et al, 1974) to equilibrium density and rate zonal ultracentrifugation (Broekman et al, 1976b). We were able to achieve with a one-step 5 hour centrifugation a separation of acid hydrolase and respiratory enzyme activities that exceeded the capabilities of the

swinging bucket method and showed that the peaks for p-nitrophenylphosphatase and lysosomal acid hydrolases were separated. We also found that after homogenization and zonal ultracentrifugation β -glycerophosphatase and aryl-sulfatase are histochemically located in vesicles that resemble rat platelet lysosomes. The importance of this finding is that it provides a basis for assessing the anatomical behavior of platelet lysosomes under various conditions, including storage.

Function of mitochondria in the intact platelet We approached the study of human platelet mitochondria by trying to isolate them using differential centrifugation, or density gradient ultracentrifugation (Broekman et al, 1974) but, in keeping with observations by Salganicoff and Fukami (1972) with swine platelets, were unable to show that such isolates had the metabolic characteristics that are shared by mitochondria from a variety of mammalian cells. We, therefore, returned to our earlier approach in which we studied mitochondrial function in the intact platelet (Cohen and Wittels, 1970) by exposure of platelet suspensions to graded decrements of osmolarity in the presence of known mitochondrial substrates. By reviving this methodology we showed, for the first time, that human platelets are capable of tight respiratory coupling and respond to a variety of mitochondrial substrates in a way that mammalian mitochondria from other cells are expected to behave (Pickett and Cohen, 1976). The new data provide a basis for studying the effects of storage on mitochondrial function in the intact platelet.

I think that several of these projects have relevance to the contract research on platelet transfusion and storage that is supported by the Surgical Research Division of the Surgeon General's Office. The quest for the lesion of platelet storage can now be pursued by applying the new techniques in studies that compare 1) the lipase activities 2) the consumption of O_2 (and the generation of compounds in the prostaglandin pathway) 3) the morphological and biochemical behavior of lysosomes and alpha granules and 4) the function of mitochondria, in stored versus fresh intact platelets. In another connection, the novel approach to the study of the metabolism of blood vessels in vivo may be useful in research on the metabolic effects of trauma and repair of blood vessels, pursuits that are of particular concern to military surgeons.

I very much appreciated the Army's interest in and support of the described studies.

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